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Development of Lithium-Induced Nephrogenic Diabetes Insipidus Is Dissociated from Adenylyl Cyclase Activity

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In antidiuresis, vasopressin (AVP) occupation of V2 receptors in renal collecting ducts activates adenylyl cyclase, resulting in increased intracellular cAMP levels, which activates protein kinase A (PKA). PKA phosphorylates both the cAMP responsive element binding protein, which induces aquaporin-2 (AQP2) transcription, and AQP2, which then is translocated to the apical membrane, allowing urine concentration. Lithium treatment often causes nephrogenic diabetes insipidus (NDI), which coincides with decreased AQP2 expression and which generally is ascribed to reduced adenylyl cyclase activity. However, the underlying mechanism by which lithium causes NDI is poorly understood. This study demonstrated that the mouse cortical collecting duct mpkCCD_{c14} cells are a good model; the deamino-8 D-arginine vasopressin (dDAVP)-induced endogenous AQP2 expression and plasma membrane localization was time-dependently reduced by treatment with clinically relevant lithium concentrations. Lithium did not affect AQP2 stability but decreased its mRNA levels. Surprisingly, the effect of lithium was cAMP independent; it did not alter AVP-stimulated cAMP production or PKA-dependent phosphorylation of AQP2 or cAMP responsive element binding protein. *In vivo*, kidney tissue of rats with lithium-induced NDI indeed generated less dDAVP-induced cAMP than that of controls, but this could be due to elevated blood AVP levels in rats with lithium-induced NDI. Indeed, Brattleboro rats, which lack endogenous AVP, with clamped blood dDAVP levels, showed no difference in dDAVP-generated cAMP generation between kidneys of rats with lithium-induced NDI and control rats. In conclusion, the first proper cell model to study lithium-induced NDI was developed, and it was demonstrated that the lithium-induced downregulation of AQP2 and development of NDI occur independent of adenylyl cyclase activity *in vitro* and *in vivo*.

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Lithium is the drug of choice for treating bipolar disorders and is prescribed to 1 in 1000 of the population (1,2). It also is currently under consideration as a therapeutic agent for alcoholism, Alzheimer's disease (3), schizoaffective disorders and AIDS (4), and cluster headaches (5). In 30 to 40% of the patients who take this medication, however, lithium causes nephrogenic diabetes insipidus (NDI), a disease in which the antidiuretic effect of the hormone arginine-vasopressin (AVP) is impaired (6), and is therewith the most common acquired form of NDI. Lithium-induced NDI also exposes the patient to an increased risk for dehydration-induced lithium toxicity. At present, however, it is still unclear how lithium affects the AVP-induced water reabsorption in the kidney.

In the normal regulation of water homeostasis, hypovolemia or hypernatremia causes the release of AVP from the posterior pituitary into the circulation (for review, see [7]). In the kidney, AVP binds its vasopressin type 2 receptor (V2R), which is

located in the basolateral membranes of the collecting duct principal cells. V2R occupation causes activation of adenylyl cyclase and a subsequent rise in intracellular cAMP levels. The increased cAMP concentration activates protein kinase A (PKA), which has at least two targets involved in the regulation of the aquaporin-2 (AQP2) water channel. First, PKA phosphorylates AQP2 itself, which thereby is translocated from vesicles to the apical plasma membrane to allow the reabsorption of water from the pro-urine. Second, cAMP increases AQP2 transcription through phosphorylation of the cAMP responsive element binding protein (CREB), which then binds and activates the AQP2 gene promoter (8,9).

Lithium-induced NDI is associated with decreased AQP2 protein and mRNA levels, attenuated apical targeting of AQP2 in rats (10–12), and decreased urine AQP2 excretion in humans (13). As isolated collecting ducts or kidneys of rats that are treated long term with lithium show a decreased generation of cAMP in response to AVP (14–17) and lithium interfered with the activation of adenylyl cyclase in renal LLC-PK₁ cells (18), it is generally agreed that lithium-induced NDI is due to reduced AQP2 levels caused by the inhibition of adenylyl cyclases by lithium (1). However, partly because of the lack of a renal principal cell line that expresses endogenous AQP2 in response to AVP, the mechanism by which lithium causes NDI is poorly understood. Recently, however, a mouse cortical collecting duct cell line (mpkCCD_{c14}) was generated (19,20), which has

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retained many of the hallmarks of principal cells from native epithelium, including the expression of endogenous AQP2 when stimulated with physiologic levels of AVP (19–21).

Here, we show that this cell line is an ideal model to study the mechanism underlying lithium-induced NDI and demonstrate in detail that in this cell line the administration of therapeutically relevant concentrations of lithium reduces AQP2 expression independent from cAMP generation. Moreover, our rat studies reveal that the reduced AQP2 levels at early stages of lithium-induced NDI are also cAMP independent *in vivo*.

Materials and Methods

Experimental Animals

Male Wistar rats were obtained from the University Animal Facility; Brattleboro rats were purchased from Harlan Laboratories (Harlan Sprague Dawley, Indianapolis, IN). Rats that weighed 250 to 350 g were maintained on a standard rodent diet and had free access to water before the experiment. For lithium therapy, lithium chloride was added to the food to give a concentration of 40 mmol/kg dry food for the first week and 60 mmol/kg thereafter, according to the methods of Marples *et al.* (11). All rats that received lithium had access to water *ad libitum* and a salt block. Water intake was monitored continuously. Wistar rats received lithium until development of NDI was established. Mini-osmotic pumps (ALZET, Model 2001, Durect Co., Cupertino, CA) that released 1 μ l/h for 7 d at 37°C were filled with 200 μ l of 5 ng/ μ l deamino-8 D-arginine vasopressin (dDAVP) in 0.9% sterile saline and implanted in the back of the neck of the Brattleboro rats at day 8. These were replaced at day 15 with new ones. Proper function of the pumps was revealed by the reduction of urine output and upregulation of AQP2 expression (see Results). For the last 24 h of the experiment, the rats were housed in metabolic cages to measure water intake and urine output and osmolality. All animal treatments were reviewed and approved by the Animal Experiments Committee of the Radboud University Medical Centre.

Tissue Preparation

Rats were anesthetized by isofluorothane, after which blood was removed by heart puncture. The rats then were killed by cervical dislocation, and the kidneys were removed rapidly. The inner medulla, outer medulla, and cortex were dissected out, and similar-sized tissue samples were minced using a scalpel. One kidney was used to make samples for immunoblotting; the other was used for drug treatment and cAMP analysis.

For immunoblotting, the tissue was homogenized in 1 ml of ice-cold sucrose dissecting buffer (300 mM sucrose, 25 mM imidazole, 1 mM EDTA, and protease inhibitors) and cleared from nuclei and unbroken cells by centrifugation at $4000 \times g$ for 15 min and diluted to 0.5 to 1 μ g/ μ l in Laemmli buffer. For drug treatment and cAMP analysis, the tissue was suspended in 1 ml modified Krebs-ringer buffer (140 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$, 10 mM NaOAc, 20 mM Tris, 2 mM NaH_2PO_4 , and 10 mM glucose [pH 7.4]) that contained 0.001% DNase I, prewarmed to 37°C. In this buffer, tissue was incubated for 5 min in 3-isobutyl-1-methylxanthine (IBMX) at 37°C, followed by an addition of 1 nM (Wistar rats) or 1 μ M (Brattleboro rats) dDAVP for 10 min at 37°C. The tissue was spun down at $800 \times g$ and lysed in 300 μ l of 0.1 M HCl for 15 min at room temperature. Leftover tissue was spun down, and the supernatant was used in a colorimetric competitive immunoassay to measure cAMP (Sigma, St. Louis, MO) and was analyzed by PAGE and Coomassie brilliant blue staining to normalize for the amount of lysed tissue.

Blood and Urine Analyses

From the collected blood, serum was prepared by incubation in the cold, followed by brief centrifugation at $600 \times g$ while urine was spun down to remove sediment. Both serum and urine samples were analyzed for osmolality and sodium, potassium, lithium, creatinine, and urea concentrations by standard procedures of the General Clinical Chemical laboratory the Radboud University Nijmegen Medical Centre.

Cell Culture

MpkCCD cells (clone 14; passages 26 to 35) were grown as described previously (19). For growth on semipermeable filters (0.4 μ m pore size, Transwell; Corning Costar, Cambridge, MA), exponentially growing cells were trypsinized and seeded at a density of 1.5×10^5 cells/cm²; 1-cm² filters were used for immunoblotting, immunocytochemistry, or cAMP assays, and 4.7 cm² filters were used for RNA isolation. In all experiments, the cells remained in culture for 8 d before being analyzed. Unless stated otherwise, the cells were treated for the last 96 h with 1 nM dDAVP to only the basolateral side to induce maximum AQP2 expression. Lithium (1 or 10 mM) was administered to the apical side, and 1 mM lithium was administered to the basolateral side. Cycloheximide (50 μ M), IBMX (0.25 mM), and ATP (10 μ M) were administered to both the apical and the basolateral sides for the indicated time points.

cAMP Assay

cAMP levels were assayed using a direct cAMP enzyme immunoassay kit (Sigma). MpkCCD cells were filter seeded and drug treated as described above. After drug treatments, filters were excised rapidly from their plastic supports, immersed in 150 μ l of 0.1 M HCl, and incubated for 15 min at room temperature. The samples then were centrifuged at $600 \times g$ for 5 min at room temperature; 100 μ l of the supernatant was drawn off and used directly in the assay, which was done according to the manufacturer's instructions. Data are based on at least two independent experiments in which triplicates per condition were taken.

Immunoblotting and Immunocytochemistry

MpkCCD cells from the 1-cm² filter were lysed in 200 μ l of Laemmli buffer, and 15- μ l samples were analyzed while 5 to 10 μ g of kidney material was analyzed. PAGE, blotting, and blocking of the polyvinylidene difluoride membranes were done as described previously (22). Membranes were incubated overnight with 1:3000-diluted affinity-purified rabbit AQP2 antibodies (23), 1:250-diluted affinity-purified rabbit anti-phosphorylated AQP2 antibodies (gift from Dr. S. Nielsen, Aarhus University, Aarhus, Denmark), 1:100,000-diluted mouse anti-tubulin antibodies (gift from Dr. Kreis, University of Geneva, Geneva, Switzerland), or 1:2000-diluted rabbit anti-CREB and p-CREB antibodies (Sigma) in TBS-T supplemented with 1% nonfat dried milk. Blots were incubated for 1 h with 1:5000-diluted goat anti-rabbit IgG or 1:2000 goat anti-mouse IgG (Sigma) as secondary antibodies coupled to horseradish peroxidase. Proteins were visualized using ECL (Pierce, Rockford, IL). Immunocytochemistry was done as described previously (24). Data are based on at least two independent experiments in which triplicates per condition were taken.

Northern Blotting

Total RNA was isolated from filter-seeded mpkCCD cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Parallel to a two-fold dilution series of a known RNA sample for semiquantification, 20 μ g of RNA of the experimental

conditions was subjected to a 1% agarose-formaldehyde electrophoresis. Subsequent Northern blotting was performed as described previously (25). The blot was hybridized with full-length [α - 32 P]dCTP-labeled probes of rat AQP2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), washed, and exposed to high-performance film (Amersham Biosciences, Uppsala, Sweden). Data are based on three independent experiments in which duplicates per condition were taken.

Statistical Analyses

Films were scanned using a Bio-Rad (Hercules, CA) 690c densitometer and analyzed using Bio-Rad software. Two-fold dilution series of protein or mRNA were blotted in parallel to allow semiquantification. After densitometric analysis of the Western or Northern blots, samples were corrected for differences in loading by being normalized to the corresponding amounts of tubulin or GAPDH (in arbitrary units), respectively. Statistical comparisons were made using the paired *t* test. *P* < 0.05 was considered significant.

Results

Lithium Causes Downregulation of AQP2 Expression in mpkCCD Cells

For determination of the point at which maximal expression of AQP2 in mpkCCD cells was reached, confluent monolayers were incubated with 1 nM dDAVP for 0, 24, 48, 72, and 96 h and lysed. Subsequent immunoblot analysis showed no AQP2 expression in unstimulated cells, whereas cells that were incubated with dDAVP showed a time-dependent increase in the

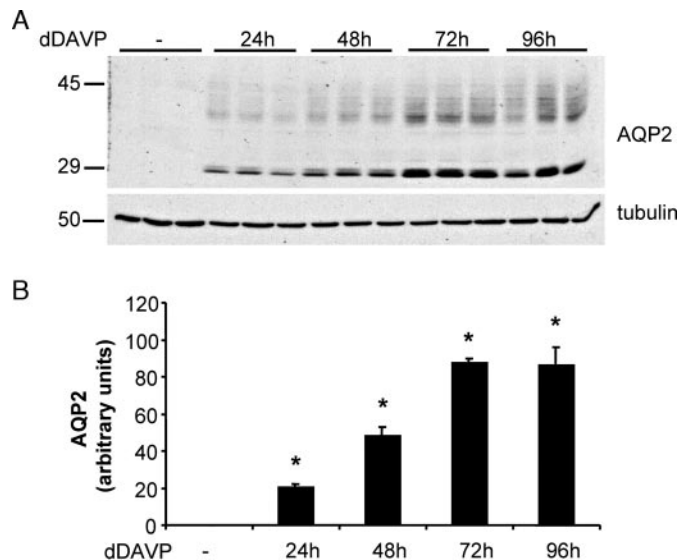


Figure 1. Aquaporin-2 (AQP2) expression in mpkCCD cells by deamino-8 D-arginine vasopressin (dDAVP) is time dependent. (A) MpKCCD cells were grown to confluence, treated for the indicated times with 1 nM dDAVP, and subjected to immunoblotting for AQP2 or tubulin (indicated). Molecular masses (in kD) are indicated on the left. Complex-glycosylated (35 to 45 kD) and nonglycosylated (29 kD) forms of AQP2 are detected. (B) The signals from A were densitometrically quantified and AQP2 signals were normalized for tubulin. Mean values of normalized AQP2 expression per time point (indicated) are given in arbitrary units. Values that are significantly different (*P* < 0.05) from untreated cells (–) are indicated by *.

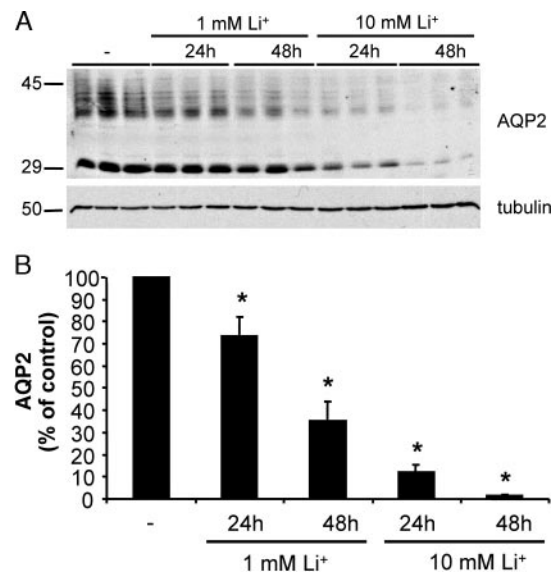


Figure 2. Effects of lithium on dDAVP-stimulated AQP2 expression in mpkCCD cells. (A) Confluent mpkCCD monolayers were treated for a total period of 96 h with 1 nM dDAVP in the absence (–) or presence of lithium (Li⁺). Cells were incubated with 1 mM lithium at the basolateral side and 1 or 10 mM lithium (indicated) at the apical side for the last 24 or 48 h (indicated). Cells were lysed and immunoblotted for AQP2 or tubulin. Molecular masses (in kD) are indicated on the left. (B) Semiquantification of the AQP2 signals and statistical analysis were done as described in the legend of Figure 1. Mean normalized AQP2 expression values are given as a percentage of the control (–) sample.

expression of unglycosylated (29 kD) and complex glycosylated (35 to 45 kD) AQP2 (Figure 1A). Immunoblotting for tubulin revealed that equivalent cell samples were loaded. Semiquantification of the signals (Figure 1B) revealed that AQP2 expression stabilized after 72 h of dDAVP treatment. For exclusion of variable AQP2 expression as a result of dDAVP incubation times, all subsequent experiments were done for a total period of 96 h of stimulation with dDAVP.

In patients who undergo lithium therapy, 1 mM serum and 1 to 10 mM urinary concentrations of lithium are commonly observed (1). Therefore, to test whether lithium affects AQP2 expression, we treated mpkCCD cells with or without lithium for the last 24 or 48 h within the dDAVP treatment. Lithium was administered in 1 mM concentration to both the basolateral and the apical compartments or 1 mM basolateral and 10 mM apical, which is further referred to as 1 or 10 mM lithium, respectively. Immunoblotting and semiquantification revealed that lithium treatment resulted in a time- and dose-dependent reduction in AQP2 expression, with an almost complete downregulation of AQP2 with 10 mM lithium for 48 h (Figure 2). Cytotoxic effects of lithium could be excluded, because the transepithelial resistance of the monolayer was not affected (data not shown) and tubulin expression levels were similar for all samples (Figure 2A). Altogether, these data indicated that mpkCCD cells are a valuable cell model to investigate the cellular mechanisms underlying lithium-induced NDI.

Lithium Treatment Alters the Cellular Localization of AQP2 in mpkCCD Cells

To establish the effects of lithium on the cellular redistribution of AQP2, we subjected lithium-treated mpkCCD cells to immunocytochemistry and confocal laser scanning microscopy. Consistent with an earlier study (19) and in agreement with the need of dDAVP for AQP2 expression, no AQP2 could be detected in untreated cells (Figure 3a), whereas cells that were stimulated for 96 h with 1 nM dDAVP showed AQP2 expression at the apical and basolateral membranes (Figure 3b). In cells that were co-treated with 1 mM lithium for the last 24 h, plasma membrane staining of AQP2 was decreased, whereas the intracellular AQP2 staining was increased (Figure 3c). This effect was even more pronounced with cells that were co-treated with 1 mM lithium for 48 h or 10 mM lithium for 24 or 48 h (Figure 3, d through f). In the latter situation, AQP2 plasma membrane staining was virtually absent.

Lithium Does not Alter the Stability of the AQP2 Protein

AQP2 downregulation can be due to increased degradation or decreased synthesis of AQP2 protein and/or mRNA. To examine at which level lithium exert its effect, we first studied its effects on AQP2 protein degradation. dDAVP-stimulated mpkCCD cells were treated with the protein synthesis inhibitor

cycloheximide and incubated in the absence or presence of 1 or 10 mM lithium for different periods. Immunoblotting for AQP2 revealed that with or without lithium, a reduction in AQP2 expression becomes apparent at 6 h of cycloheximide treatment, which increases at longer incubation times (Figure 4, A and B). Semiquantification of the signals after normalization for tubulin revealed that the relative reduction of AQP2 expression in time was not different between incubations with or without lithium (Figure 4C). This indicated that lithium does not decrease the stability of the AQP2 protein.

Lithium Decreases AQP2 mRNA Expression

To investigate whether lithium affects AQP2 mRNA expression, total RNA was isolated from mpkCCD cells that were treated with dDAVP for 0, 24, 48, or 96 h or in the presence of 1 or 10 mM lithium for the last 24 or 48 h. In line with AQP2 protein levels (Figure 1), subsequent Northern blotting revealed that dDAVP treatment increased AQP2 mRNA levels to a maximal level at 72 h, which was sustained at 96 h (Figure 5A). Lithium treatment seemed to affect the AQP2 mRNA levels; it resulted in a dose-dependent decrease in AQP2 (Figure 5A). As the intensity of the GAPDH signals was similar in all lanes, indicating equal loading, semiquantification of the

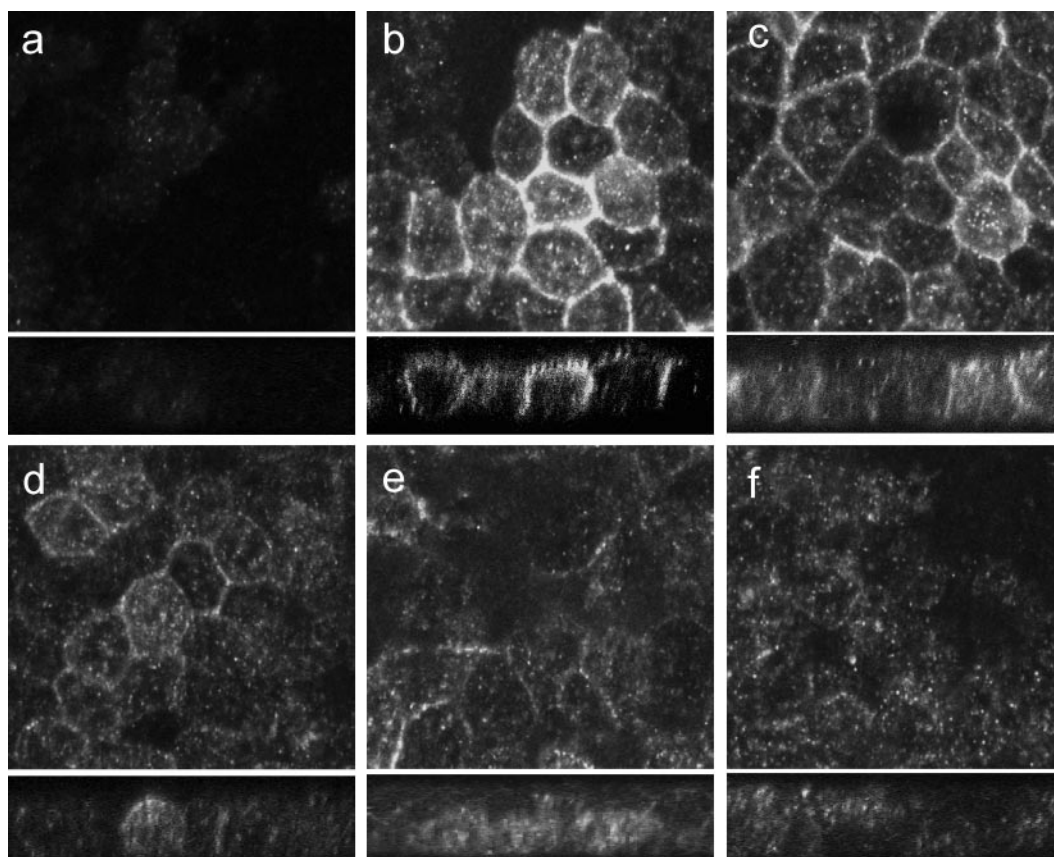


Figure 3. Effects of lithium on the cellular distribution of AQP2 in mpkCCD cells. Confluent mpkCCD monolayers were left untreated (a), treated with 1 nM dDAVP for 96 h (b), or treated with both dDAVP for 96 h and either 1 mM lithium for 24 h (c) or 48 h (d) or 10 mM lithium for 24 h (e) or 48 h (f), as described in Figure 2. Then, cells were subjected to AQP2 immunocytochemistry and analyzed by confocal laser scanning microscopy.

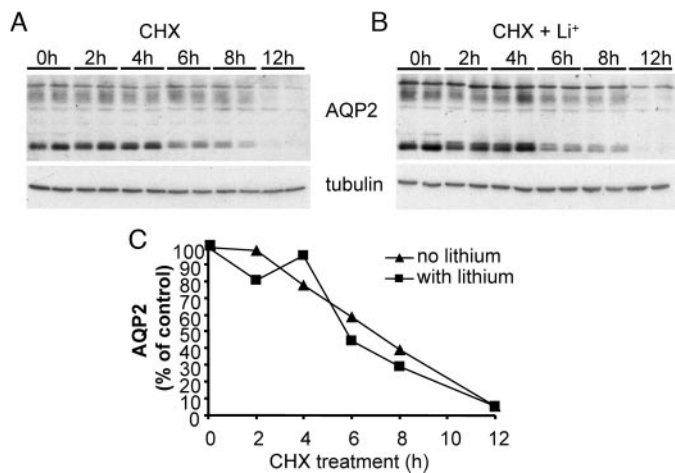


Figure 4. Effects of lithium on AQP2 protein stability in mpkCCD cells. (A and B) Confluent mpkCCD monolayers were treated with 1 nM of dDAVP for 96 h, followed by addition of 50 μ M cycloheximide (CHX), without or with 10 mM lithium ($+Li^+$) for the indicated times. (C) Semiquantification of the AQP2 signals in A and B was done as described in Figure 2.

signals and normalization for GAPDH confirmed these observations (Figure 5B).

Lithium Does not Decrease Adenylyl Cyclase Activity in mpkCCD Cells

In line with the current dogma, the lithium-induced reductions in AQP2 protein and mRNA levels could be due to decreased cAMP levels. To investigate this in mpkCCD cells, we pretreated filter-seeded cells for 24 h with 0, 1, or 10 mM lithium. During the last 10 min, the phosphodiesterase inhibitor IBMX was added to inhibit cAMP degradation, and 1 nM dDAVP was added or not for the last 2 min. Finally, the cells were lysed and subjected to cAMP assays. In the absence of lithium, dDAVP caused a four-fold increase in the cAMP concentration (Figure 6), indicating activation of adenylyl cyclase *via* the V2R signaling cascade. Surprising, however, dDAVP-stimulated cells that were pretreated with 1 or 10 mM lithium showed a cAMP accumulation that was even larger than that of cells that had not been pretreated with lithium. As a negative control, dDAVP addition in the presence of 10 μ M ATP, a known inhibitor of AVP-stimulated cAMP production in the collecting duct (26), showed a blunted cAMP accumulation compared with dDAVP alone.

Because raised intracellular cAMP levels activate PKA, which phosphorylates AQP2 and CREB, analysis of the phosphorylation state of these proteins represents another indicator of adenylyl cyclase activity. Therefore, confluent mpkCCD monolayers were treated with 1 or 10 mM lithium for 24 or 48 h, lysed, and immunoblotted for AQP2, phosphorylated AQP2, CREB, phosphorylated CREB, and tubulin (Figure 7). Lithium induced a similar downregulation of phosphorylated AQP2 and total AQP2, without affecting the levels of tubulin, used as internal standard (Figure 7A). In the absence of lithium, dDAVP caused a significant decrease in total CREB without reducing phos-

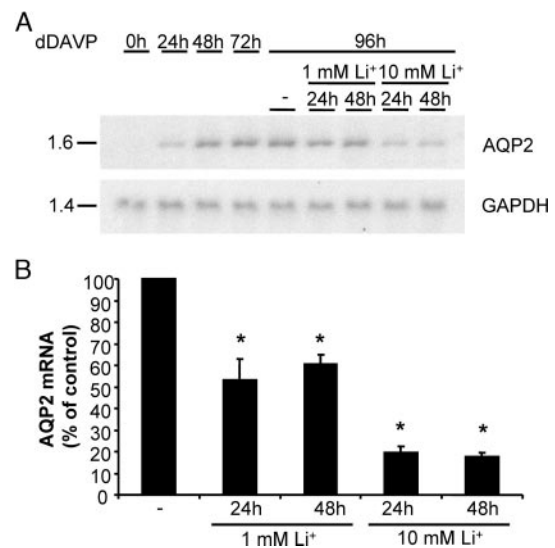


Figure 5. Effects of lithium on AQP2 mRNA levels in mpkCCD cells. (A) Confluent mpkCCD monolayers were treated with 1 nM of dDAVP for the indicated times in the absence or presence of the indicated concentrations and times of lithium (Li^+), as described in Figure 2. Subsequently, total RNA was extracted and subjected to Northern blotting using AQP2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes (indicated). The approximate lengths of the messengers (in kb) are indicated on the left. (B) For the 96-h dDAVP treatments without (–) or with lithium, AQP2 and GAPDH signals from three independent experiments were densitometrically quantified. The AQP2 mRNA levels were normalized for GAPDH. Data are given and statistically analyzed as described in Figure 2.

phorylated CREB levels ($P = 0.07$; Figure 7B). Consistent with the unaffected AQP2 phosphorylation with lithium, however, lithium did not reduce CREB phosphorylation when in the continuous presence of dDAVP (Figure 7, B and C). Altogether, these data reveal that lithium does not affect V2R-mediated activation of adenylyl cyclase in mpkCCD cells.

Lithium Does not Affect Adenylyl Cyclase Activity In Vivo

To analyze whether adenylyl cyclase activity is affected in lithium-induced NDI (*i.e.*, *in vivo*), we fed three Wistar rats food that contained lithium to induce NDI, three control rats normal diet. After 12 d, significant differences in urine volumes and osmolalities between lithium-fed (106 ± 4 ml/24 h; 146 ± 9 mOsm) and control rats (13 ± 2 ml/24 h; 457 ± 39 mOsm) indicated that lithium-induced NDI had developed. At this point, the serum lithium concentration in lithium-fed animals was 0.71 ± 0.19 mM, whereas that of control animals was below the detection limit of 0.05 mM. The rats were killed, and their kidneys were removed immediately. As anticipated, immunoblotting of membranes from one kidney inner medulla per rat for AQP2 and tubulin revealed a marked reduction of AQP2 in the lithium-treated rats (Figure 8A). The inner medullas of the contralateral kidneys were used to measure cAMP accumulation after treatment with IBMX or IBMX combined with 1 nM dDAVP. In the presence of IBMX, dDAVP increased intracellular cAMP levels nearly three-fold in the control rats, whereas

it increased cAMP levels only two-fold in lithium-fed rats (Figure 8B). Therefore, lithium treatment of rats clearly decreased dDAVP-stimulated cAMP accumulation in kidneys that were isolated from these animals.

However, in rats that had lithium-induced NDI, the blood AVP levels are elevated (27), and, as shown for numerous receptors *in vitro* (28), including the V2R (29,30), an increased receptor occupancy results in their activation, internalization, and degradation. Therefore, the decreased cAMP accumulation with renal tissue of lithium-fed rats after dDAVP treatment might be accounted for by a decreased V2R availability. To test this hypothesis, we repeated the experiment above with Brattleboro rats, which lack endogenous AVP; blood AVP levels were clamped using implanted minipumps that released continuous levels of dDAVP. Establishment of a reduced urine volume (data not shown) before the diuretic effects of lithium treatment became apparent illustrated proper release of dDAVP from the minipumps. Eighteen days after the onset of feeding rats with food with or without lithium, urine volume and osmolalities of lithium-fed rats (108 ± 18 ml/24 h; 192 ± 36 mOsm) differed significantly from those of dDAVP-infused control rats (23 ± 9 ml/24 h; 1807 ± 359 mOsm), which indicated that NDI had developed. Lithium-fed rats had a serum lithium concentration of 1.20 ± 0.23 mM, whereas that of control rats was undetectable. As anticipated, immunoblotting revealed a markedly reduced AQP2 expression in the cortex, outer medulla, and inner medulla of lithium-treated rats compared with controls (Figure 9). The dDAVP-induced cAMP accumulation after $1 \mu\text{M}$ dDAVP treatment, however, was not different for the cortex ($P = 0.21$), outer medulla ($P = 0.36$), or inner medulla ($P = 0.134$) between rats that had lithium-induced NDI and their controls. This indicated also that *in vivo* lithium does not affect

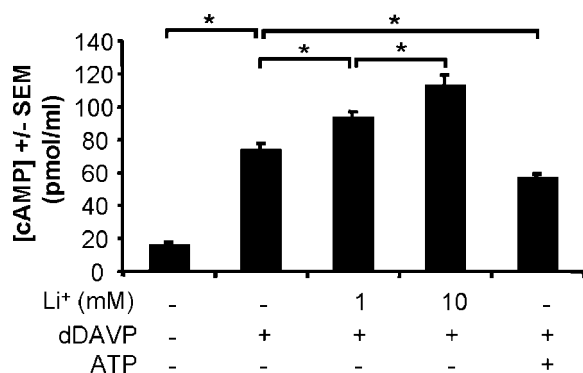


Figure 6. Effects of lithium on dDAVP-stimulated cAMP production in mpkCCD cells. MpKCCD monolayers were left untreated (–) or treated for 24 h with the indicated concentrations of lithium (Li^+), as described in Figure 2. All cells were pretreated with 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min. Where indicated (+), cells were subsequently stimulated with 1 nM of dDAVP with or without 10 μM ATP for 2 min, in the continuous presence of lithium where indicated. Cells were lysed and subjected to cAMP measurements. The mean cAMP concentration ($[\text{cAMP}] \pm \text{SEM}$ [in pmol/ml]) was determined from three independent filters per condition. Significant differences ($*P < 0.05$) between different conditions are indicated.

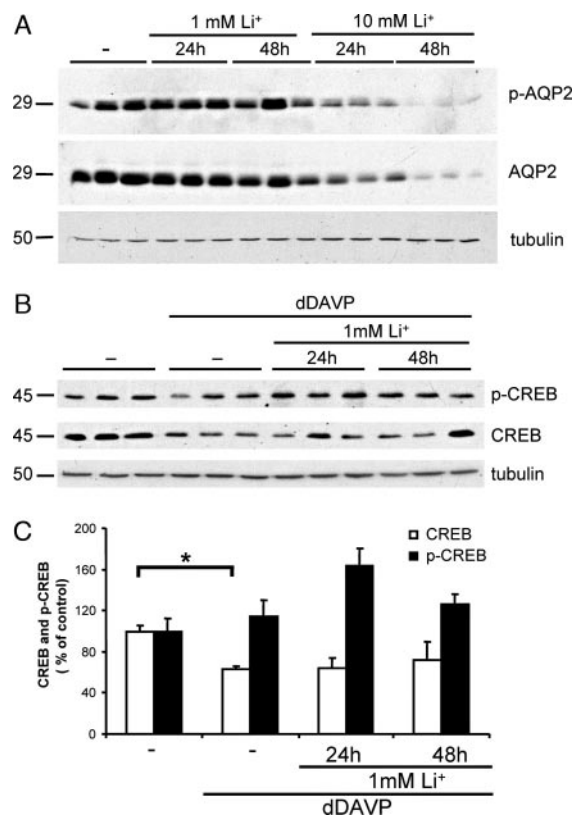


Figure 7. Effects of lithium on AQP2/cAMP responsive element binding protein (CREB) phosphorylation in mpkCCD cells. Confluent mpkCCD cells were treated for 96 h with 1 nM dDAVP in the absence (–) or presence of the indicated concentrations and times of lithium (Li^+), as described in Figure 2. Then, cells were lysed and immunoblotted for phosphorylated AQP2 (p-AQP2), AQP2, and tubulin (A) or for CREB and its phosphorylated form (p-CREB) and tubulin (B). (C) Semiquantification of the CREB and p-CREB signals and statistical analysis were done as described in the legend of Figure 1. Mean normalized CREB and p-CREB expression values are given as a percentage of the control (–) sample. Molecular masses (in kD) are indicated on the left.

cAMP generation by AVP-activated adenylyl cyclases and that the lithium-induced downregulation of AQP2 occurs independent from cAMP.

Discussion

MpkCCD Cells Represent a Proper Model to Study Lithium-Induced Downregulation of AQP2

The major handicap for deciphering the mechanism by which lithium induces NDI at the cellular level has been the lack of a cell line that expresses AQP2 endogenously in response to physiologic levels of AVP and in which the AQP2 expression is sensitive to therapeutically relevant lithium levels. We demonstrated that the mpkCCD cell line fulfills these requirements, as 1- and 10-mM concentrations of lithium cause a time- and concentration-dependent decrease in AQP2 protein levels (Figure 2), and the plasma membrane expression of AQP2 was decreased in response to lithium (Figure 3). These observations

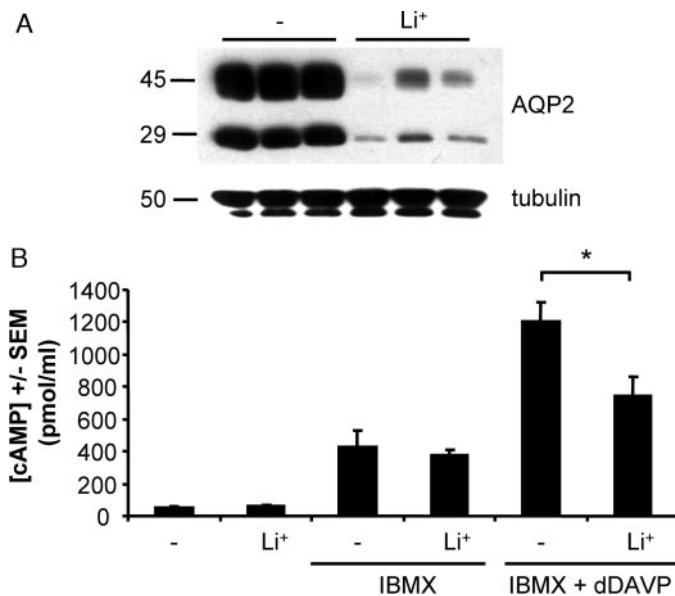


Figure 8. Effects of lithium on AQP2 expression and dDAVP-stimulated cAMP production in rat inner medulla. (A) Three Wistar rats were fed a normal diet (–), and three were fed a diet that contained lithium (Li⁺). After 12 d, lithium-induced nephrogenic diabetes insipidus (NDI) was confirmed, the rats were killed, and kidneys were removed immediately. Membranes from one kidney inner medulla per rat were solubilized and immunoblotted for AQP2 and tubulin (indicated). Molecular masses (in kD) are indicated on the left. (B) The other kidney inner medulla of each rat was minced finely and divided into three portions, which were left untreated, treated with 0.25 mM IBMX (indicated) for 10 min, or treated with IBMX followed by stimulated with 1 nM dDAVP for 10 min (IBMX + dDAVP). The tissue was lysed and subjected to cAMP measurements, and cAMP values were corrected for protein content. Values are means \pm SEM of the corrected cAMP concentration ([cAMP] [in pmol/ml]) from three rats. Significant differences (* P < 0.05) between different groups are indicated.

are in close agreement with the findings of Marples *et al.* (11), who demonstrated the correlation between the onset of lithium-induced NDI and AQP2 downregulation in rats, and the work from Dousa *et al.* (31), who showed that lithium affects microtubule assembly in the renal medulla, thereby inhibiting the AVP-induced AQP2-mediated antidiuretic effect. As lithium often results in hypercalcemia, which can cause NDI by itself, the observed downregulation of AQP2 in mpkCCD cells with lithium under normocalcemic conditions clearly reveals that lithium also affects AQP2 expression directly.

Lithium Affects AQP2 Gene Transcription in mpkCCD Cells

The lithium-induced reduction in AQP2 expression, as found in mpkCCD cells, could be due to increased AQP2 protein/mRNA degradation and/or reduced AQP2 translation/transcription. Using the protein synthesis inhibitor cycloheximide, no difference in the level of AQP2 degradation was observed with or without lithium (Figure 4). As the pulse (chase) labeling assay did not yield enough product, which is common for polarized cells, we were

not able to determine the effect of lithium on the speed of AQP2 translation. However, lithium caused a strong downregulation of the AQP2 mRNA levels (Figure 5). As the AQP2 protein levels usually follow AQP2 mRNA levels, the lithium-induced downregulation of AQP2 protein in mpkCCD cells likely originates from effects on AQP2 transcription and/or mRNA degradation. It remains to be established which factors mediate the effect of lithium on AQP2 mRNA levels.

Lithium-Induced Reduction of AQP2 Expression in Lithium-NDI is cAMP Independent

Our results reveal that both in mpkCCD cells and *in vivo*, the lithium-induced downregulation of AQP2 occurs independent from AVP-induced cAMP levels. In mpkCCD cells, preincubation with lithium did not affect the cAMP response that was generated by physiologic levels of AVP (Figure 6). Consistent with this, whereas lithium reduced dDAVP-induced AQP2 expression levels, the relative level of the cAMP-dependent AQP2 phosphorylation was not reduced (Figure 7A). In addition, lithium did not affect CREB expression levels or its level of phosphorylation (Figure 7, B and C); the latter is cAMP dependent (32). These findings do not corroborate with the observation that lithium interferes with the activation of adenyl cyclase in LLC-PK₁ cells (18). However, LLC-PK₁ cells originate from the renal proximal tubule, whereas lithium-induced NDI is caused by insufficient functioning of collecting duct principal cells. The mpkCCD cells that were used in this study are derived from principal cells, show a reduced AQP2 expression after lithium treatment, and, therefore, are a better model to study the mechanism of lithium-induced NDI.

As reported by many others (14–17) and as found in this study (Figure 8), comparing the cAMP-generating response to AVP of renal tissue from rats that had lithium-induced NDI *versus* controls suggest that lithium reduces AVP-induced cAMP generation. However, as blood AVP levels are increased in rats that had lithium-induced NDI (27), the reduced amount of generated cAMP in lithium-induced NDI renal tissue could just be due to a reduced number of available V2R, which is a common negative-feedback response of cells to increased agonist levels (28). Indeed, when the effects of a difference in endogenous AVP levels were excluded by implanting dDAVP-filled minipumps into AVP-deficient Brattleboro rats, differences in dDAVP-induced cAMP generation in the renal cortex, outer medulla, and inner medulla of rats that had lithium-induced NDI and control rats were no longer observed, whereas the AQP2 levels clearly were reduced in the rats that had lithium-induced NDI (Figure 9). Of note is that the obtained plasma lithium levels were higher in the Brattleboro rats than in the Wistar rats, which underscores our hypothesis that the cause by which lithium causes NDI is cAMP independent, as then one would expect to see a more pronounced difference in generated cAMP between the lithium and nonlithium groups of Brattleboro rats than of Wistar rats.

Also, to clamp blood vasopressin levels in the Brattleboro rats, we used minipumps that released dDAVP, giving a submaximal level of urine-concentrating ability (33). Because the obtained blood dDAVP levels in these rats likely were in-

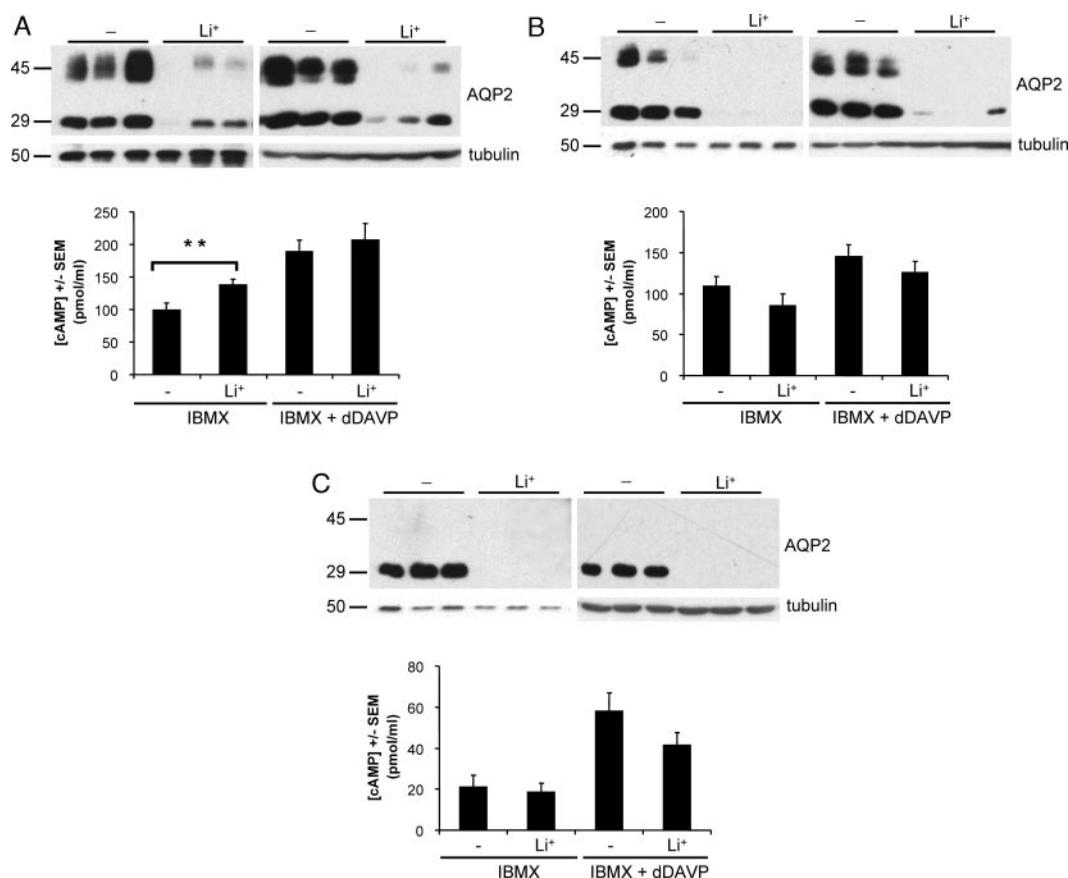


Figure 9. Effects of lithium on AQP2 expression and dDAVP-stimulated cAMP production in kidneys of dDAVP-clamped rats. Six Brattleboro rats were fed a normal diet (–), and six were fed a diet that contained lithium (Li⁺). From day 8 onward, dDAVP-filled minipumps were implanted in all rats. On day 18, lithium-induced NDI was confirmed, the rats were killed, and kidneys were removed immediately. Membranes from one kidney inner medulla (A), outer medulla (B), and cortex (C) per rat were solubilized and immunoblotted for AQP2 and tubulin (indicated). The other kidney inner medulla (A), outer medulla (B), and cortex (C) of each rat was minced finely, divided into two portions, and analyzed for ability to generate cAMP in response to dDAVP as described in Figure 8, except that here 1 μ M dDAVP was used. Values and statistical analysis were done as described in the legend of Figure 8.

creased compared with our Wistar rats and the level of receptor occupancy by an agonist (here AVP/dDAVP) and, consequently, its removal from the cell surface depends on the concentration of the agonist, the cAMP-generating ability of Brattleboro rat kidney homogenates had to be tested with a higher dDAVP concentration than used with Wistar rats. Considering that dDAVP cannot be measured using the standard assays, 1 μ M dDAVP seemed to be a well-chosen concentration because it generated moderate cAMP responses within the range for sensitive detection of differences (10 to 200 pmol/ml). One could question whether this higher concentration of dDAVP would affect our results. Although we cannot exclude this possibility, this is unlikely for several reasons. First, dDAVP activates the V2R and V1b receptor at high potency, but in the rat kidney, only V1a and V2R are expressed (34,35). Second, 1 μ M of dDAVP does not seem to activate V1aR, because treatment of V1aR-expressing rat brain septum tissue with 1 or even 10 μ M dDAVP did not result in an increased inositol-phosphate production (36). Third, the control and lithium-induced NDI Brattleboro groups were similarly infused with dDAVP. Together, we believe that these and our mpkCCD

cell line data strongly indicate that lithium-induced downregulation of AQP2 and thus lithium-induced NDI occur independent from adenylate cyclase activity. Our findings are in line with the finding that in rats that were treated for only 7 d with lithium, their lithium-induced polyuria was not interrupted by low doses of AVP but also not by cAMP (37).

Lithium-Induced NDI Development

On the basis of our data and those of others, lithium-induced NDI might develop as follows: During the onset of lithium-induced NDI development (approximately 10 d in rats), collecting duct morphology is not changed, but AQP2 protein levels are decreased (38). Our data are a model for this early onset, as the mpkCCD cells were treated with lithium for a maximum of 48 h and did not show a change in the number of cells or appearance of the intercalated cell marker H⁺-ATPase (data not shown; see below). Also, our rats were treated for a maximum of 18 d with lithium and had a normal collecting duct morphology (data not shown). Our data indicate that the lithium-induced downregulation of AQP2 in this period is cAMP and adenylate cyclase independent and is due to the reduction of

AQP2 mRNA. Presumably as a result of the toxic effect of lithium on principal cells, chronic lithium therapy (>4 wk in rats) further results in a decreased number of principal cells to the benefit of intercalated cells (39), which also will contribute to a reduced dDAVP-induced cAMP generation in kidneys of rats that had lithium-induced NDI. Recently, cyclo-oxygenase 2 (COX2) expression was suggested to be involved in lithium-induced NDI (40), but this was debated by others (41). Although our study does not exclude a role of COX-2 in development of lithium-induced NDI, the lithium-induced AQP2 downregulation in our mpkCCD cells, which are grown in the absence of COX2-producing interstitial cells, indicate that COX2-derived prostaglandins are not necessary for lithium-induced NDI development.

Although highly debated (42,43), it needs to be noted that several studies revealed that abnormalities of cAMP signaling in the brain underlie bipolar disorders (44). Also, lithium treatment for >1 wk reduced receptor-mediated cAMP formation through inhibition of a Ca^{2+} /calmodulin-sensitive adenylyl cyclase and diminished phosphorylation of the CREB transcription factor in brain tissue and cells (2,45,46). As such, our data that the onset of lithium-induced NDI is cAMP independent might not be of relevance to the brain.

Conclusion

Our *in vitro* and *in vivo* data indicate that we have established the first appropriate cell model to unravel the mechanism underlying lithium-induced NDI; that lithium-induced downregulation is due to diminished AQP2 mRNA levels, likely through reduced AQP2 gene transcription; and that the onset of lithium-induced AQP2 degradation and NDI development occurs independent from the activity of principal cell adenylyl cyclase activity. As such, it resets the scope of the cellular signaling pathway(s) by which lithium causes AQP2 downregulation and NDI development. Several studies in brain report that the positive effect of lithium in bipolar disorders might be mediated through inhibition of glycogen synthase kinase 3 and/or phospho-inositol kinases (42,43). It remains to be established whether these pathways and proteins underlie lithium-induced NDI. Future studies will focus on the identification of the pathways and proteins that are involved in lithium-induced NDI, for which mpkCCD cells seem to be an ideal model system and may become important pharmacologic targets to reduce development of NDI in lithium-treated patients with bipolar disorders.

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